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Structures of sulfated oligosaccharides isolated from the respiratory mucins of a non-secretor (O, Le^{a+b-}) patient suffering from chronic bronchitis

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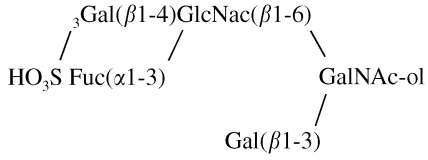
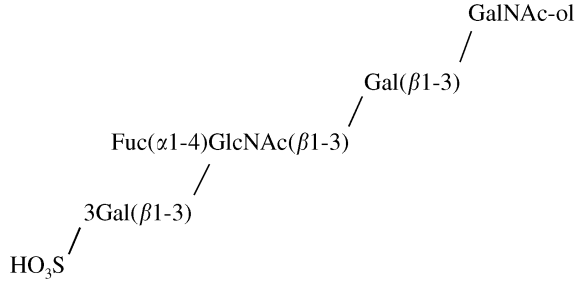
Mucin glycopeptides were prepared from the respiratory mucus of a non-secretor, chronic bronchitic patient with blood group O, Le^{a+b-}. Oligosaccharides were released by alkaline borohydride treatment and purified by anion-exchange chromatography, size-exclusion chromatography and high performance anion-exchange chromatography. Structural studies employed 400-MHz ^{1}H-NMR spectroscopy and matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). Nine monosulfated oligosaccharides ranging in size from tetra- to hexasaccharide, were fully characterized in this study. The sulfate group occurs either on the C-3 of a terminal galactose residue or on the C-6 of a N-acetylglucosamine residue. In keeping with the non-secretor status of the patient, no structure with an (*α*1-2)-linked fucose residue was found. Five of the structures had fucose present in (*α*1-3)-linkage in the X determinant, while only one oligosaccharide (compound 7b) was seen with fucose (*α*1-4)-linked in the Le^a determinant. Eight structures isolated from the mucins of the non-secretor patient had not been found previously in the respiratory mucins; they are listed below.

7a



7b



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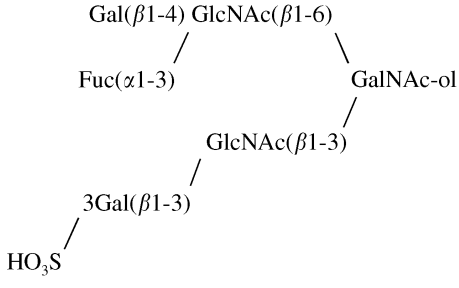

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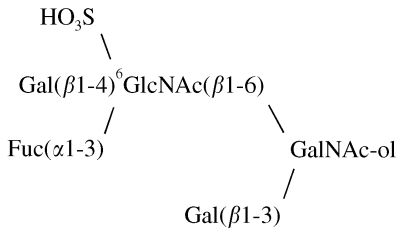

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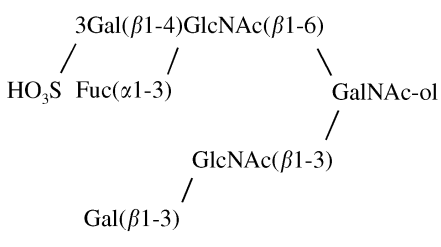

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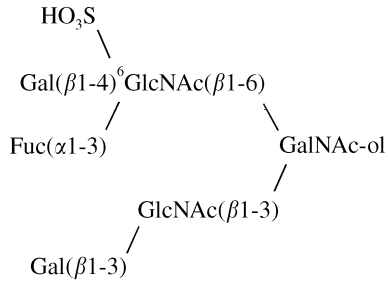

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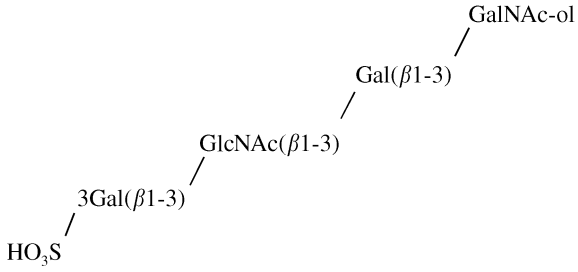

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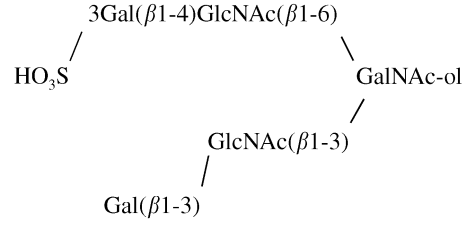

***Keywords*: chronic bronchitis, MALDI-MS, mucins, ^{1}H-NMR, non secretor**

Introduction

Human respiratory mucus forms a protective barrier over the bronchial epithelium against inhaled particles and microorganisms. Its major components are mucins which are 70–80% glycosylated. The majority of the oligosaccharides are acidic; they contain either sialic acid or sulfate, or both. As such, they are negatively charged under physiological conditions, and the charges have been implicated in maintaining the three-dimensional, rod-like structure typical of mucins. Not only are the carbohydrate chains instrumental in the physicochemical properties of mucins, but they also represent the sites of attachment for bacteria that cause airway infections and other pathological situations. Evidence exists that sulfate group is involved in this biological function as well [1, 2].

Structural characterization of the respiratory mucin oligosaccharide chains is a prerequisite to gaining further insight into their functions. This study deals with the purification and structural characterization of nine low-molecular-mass, monosulfated oligosaccharides isolated from the sputum of a non-secretor patient with blood group O suffering from chronic bronchitis. The oligosaccharide-alditol structures are compared with the neutral and the monosialyl oligosaccharides from the mucins of the same non-secretor individual [3, 4], to monosialyl oligosaccharides obtained from secretor individuals with cystic fibrosis [5–7] or bronchiectasis [8] and to monosulfated oligosaccharides obtained from secretor individuals with cystic fibrosis [7, 9]. Previous reports have suggested that sulfate could be localized on the C-6 or C-4 of galactose or on the C-6 of *N*-acetylglucosamine residue from carbohydrate chains of cystic fibrosis respiratory mucins [10–12]. Lamblin *et al.* [9] have already found that sulfate could be localized on the C-3 of galactose and Lo-Guidice *et al.* [7] on the C-3 of galactose and on the C-6 of *N*-acetylglucosamine. In the present study, we describe a series of 9 oligosaccharides where sulfate is localized either on the C-3 of galactose or on the C-6 of N-acetylglucosamine.

Materials and methods

Isolation of bronchial oligosaccharide-alditols

The collection of sputum from a chronic bronchitic patient W. and the subsequent preparation of bronchial mucus glycopeptides from it have been described previously [3]. Alkaline borohydride reductive degradation of bronchial glycopeptides (fraction F2) was performed as described previously [13]. The resulting mixture of glycopeptides and reduced oligosaccharides was applied to a Dowex AG1X2 column [13]. Four fractions were obtained: fraction I (neutral), fraction II (sialylated), and fractions III and IV (sialylated and sulfated oligosaccharides). Fraction III was subfractionated by gel filtration on a Bio-Gel P4 column into high-, medium- and low-molecular-mass compounds as described previously [13]. A pool of low-molecular-mass (average $M_r < 1000$) oligosaccharide-alditols (IIIC) was obtained by this procedure.

Further fractionation of pool IIIc was carried out by HPAEC, using a column (4×250 mm) of pellicular resin Carbopac PA 100 (Dionex Corp., Sunnyvale CA) and a PAD 2-pulsed amperometric detector (Dionex Corp., Sunnyvale CA). The chromatographic data were integrated and plotted using a spectra-Physic model Sp 4270 integrator (San Jose, CA). Elution of oligosaccharide-alditols contained in fraction IIIc was performed at alkaline pH, at a flow rate of 1 mL min^{-1}, in 0.1 M NaOH for 10 min followed by a linear gradient of sodium acetate to 0.1 M NaOH, 0.05 sodium acetate at 16 min, to 0.1 M NaOH/0.07 M sodium acetate at 35 min and to 0.1 M NaOH/0.4 M sodium acetate at 80 min [7].

An anion micro membrane suppressor (Dionex Corp., Sunnyvale CA) was connected to the detector's outflow in order to partially remove Na^+ ions from the effluents of the column. By this procedure sodium acetate was converted to acetic acid and sodium hydroxide to water. The acetic acid, produced by this method, was neutralized by ammonium bicarbonate [7].

MALDI-MS

The oligosaccharide-alditols were analysed by matrix assisted laser desorption (MALDI) on a Vision 2000 time of

flight mass spectrometer equipped with a reflector and a 330 nm UV Laser. The analytes were dissolved in water at a concentration of approximately 40 picomoles per microlitre. One microlitre of this solution was then mixed with one microlitre of a matrix solution, either matrix A: 2,5-dihydroxybenzoic acid (10 mg mL^{-1}) in methanol : water (70:30 (v/v)), or matrix B: 3-aminoquinoline (10 mg/mL) in ethanol : water (30:70 (v/v)). Each spectrum is the sum of 25 accumulated shots.

^{1}H-NMR spectroscopy

The HPAEC-fractionated oligosaccharide-alditols were repeatedly treated with D_2O (Aldrich; 99.8% then 99.96%) at room temperature and pD $\approx$ 6, with intermediate lyophilization. Prior to ^{1}H-NMR spectroscopic analysis, the samples were redissolved in 0.4 mL of D_2O (99.96 atom % D, Aldrich) and filtered through cotton-Wool into 5 mm NMR tubes (Wilmad; 535-PP). The 400 MHz ^{1}H-NMR spectroscopy was performed on a Brucker AM-400 WB spectrometer, operating under the control of an Aspect 3000 computer (Centre Commun de Mesures, Université des Sciences et Techniques de Lille, Villeneuve d'Ascq, France). The probe temperature was kept at 27.0 °C ($\pm$ 0.1). Chemical shifts (δ) are expressed in parts/million (ppm) downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured by reference to internal acetone ($\delta = 2.225$ ppm in D_2O at 27 °C), with an accuracy of 0.002 ppm. In all the figures, the resonances marked by an asterisk (*) stem from frequently occurring non-protein, non-carbohydrate contaminants. The two-dimensional homonuclear COSY experiment was performed by use of the standard Bruker pulse program COSY.

Results

Sputum was collected from a 63 year-old male patient who suffered from chronic bronchitis as a result of cigarette smoking [3]. The sputum was not purulent. The patient's blood group was typed O, Le^{a+b-}. The mucin glycopeptides did not exhibit any H antigenic activity, thus, the patient was identified as non-secretor [3].

Isolation and purification of monosulfated oligosaccharide-alditols

The respiratory mucin glycopeptides were prepared as previously described [3]. The chemical composition of the glycopeptides (fraction F2) has been reported previously [3]. Fraction F2 (632 mg) was submitted to alkaline borohydride degradation and then fractionated by ion-exchange chromatography into fractions I–IV [3]. Fraction III which was eluted from Dowex AG1 $\times$ 2 with H_2SO_4 0.025 M, was subfractionated into IIIa, IIIb and IIIc by chromatography on Bio-Gel P4 (Fig. 1). Fraction IIIc

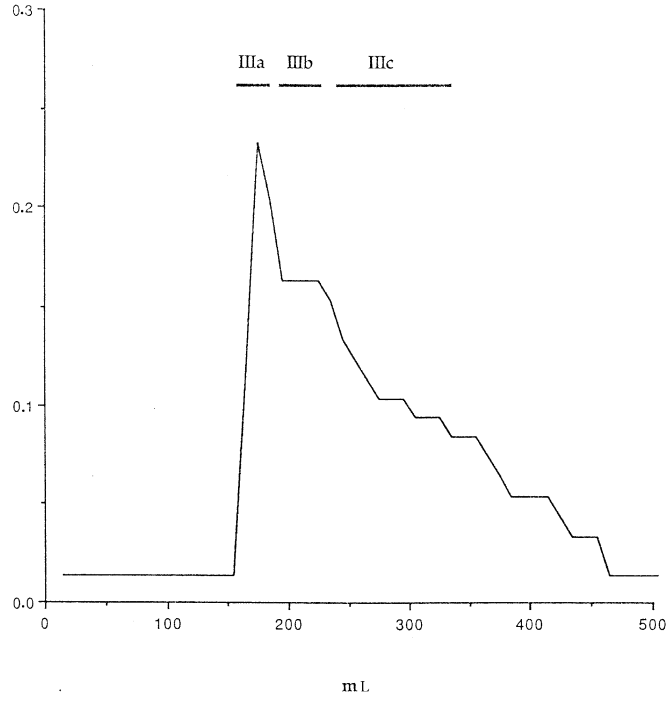

Figure 1. Bio-Gel P4 (200–400 mesh) elution profile of fraction III obtained after alkaline borohydride reductive treatment of mucin glycopeptides (fraction F2) and ion-exchange chromatography. The column (2 $\times$ 100 cm) was eluted with 0.1 M acetic acid. Aliquots were analysed for neutral sugars [5].

(6 mg), containing small-sized, acidic oligosaccharide-alditols [5, 7, 14] was further purified by HPAEC on a Carbopac PA 100 column using gradient B, described by Lo-Guidice *et al.* [7]. With this gradient, 41 peaks were obtained (Fig. 2). The nine main peaks were studied for structure determination (IIIc-6, IIIc-7, IIIc-8, IIIc-12, IIIc-17, IIIc-20, IIIc-23′, IIIc-25 and IIIc-29). Peak IIIc-6 was studied by MALDI-MS analysis and showed the presence of a $(M + 2Na\text{-}H)^+$ at m/z 1021 (Table 1), which may correspond to a sulfated pentasaccharide-alditol with GalNAc-ol, Gal, GlcNAc, Fuc and sulfate group in the molar proportion of 1:2:1:1:1. The ^{1}H-NMR spectrum showed the presence of two isomeric structures, but it was difficult to assign the different signals and the amount of this fraction was in too small quantity to confirm the structures by a II-D COSY experiment. The structures of the oligosaccharides-alditols contained in the eight other main peaks (IIIc-7, IIIc-8, IIIc-12, IIIc-17, IIIc-20, IIIc-23′, IIIc-25 and IIIc-29) were determined, the other peaks being heterogeneous and containing low amount of carbohydrates were not studied further.

The primary structures of the acidic oligosaccharide-alditols obtained from respiratory mucins of this patient suffering from chronic bronchitis were established by combining the results of MALDI-MS and ^{1}H-NMR spectroscopy.

Oligosaccharide-alditols with a core type or a core type 2

Five structures were identified in this category; their ^{1}H-NMR chemical shift values are listed in Table 2. Core type 1 is defined by the chemical shifts $\delta \approx 4.39$–4.40 for GalNAc-ol H-2 and $4.13 < \delta < 4.19$ for GalNAc-ol H-5 [15]. Core type 2 is defined by the chemical shifts $\delta = 4.39$ for GalNAc-ol H-2 and $4.26 < \delta < 4.29$ for GalNAc-ol H-5 [15].

*Structure **IIIc-25***

MALDI-MS analysis shows the presence of an ion $(M + 2Na\text{-}H)^+$ at m/z 875 (Table 1) (Fig. 3), which may correspond to a sulfated tetrasaccharide-alditol with GalNAc-ol, Gal, GlcNAc and sulfate group in the molar proportion of 1:2:1:1. The ^{1}H-NMR spectrum showed the occurrence of a pure compound (Fig. 4). The NMR parameters of compound IIIc-25 are listed in Table 2. The value $\delta = 4.556$ for H-1 is a new parameter for $Gal^{3,3,3}$ showing that this galactose residue is substituted by a sulfate group in position 3, as shown by the shift for H-1 and H-4 of this residue (Table 2). These downfield shift resonances are attributable to the C-3 substitution by a sulfate residue of this $Gal^{3,3,3}$ residue [9]. It is very clear that it is an extension of the neutral tetrasaccharide 9Ba described in Lhermitte *et al.* [3], and of the structure '5' obtained from ovarian cyst mucins, described by Mustaers *et al.* [16]. This compound IIIc-25 corresponds to a sulfated derivative of 9Ba. It has the following primary structure:

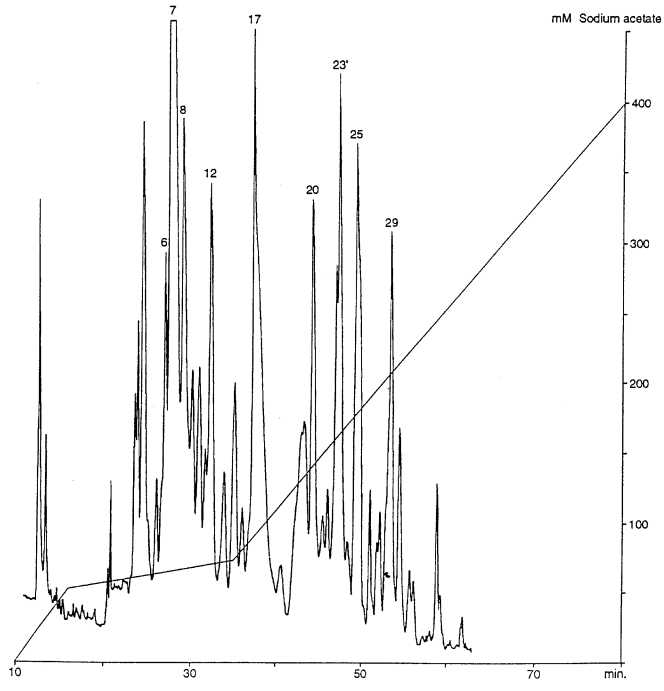

Figure 2. HPAEC elution profile of mucin oligosaccharide-alditols of fraction IIIc on CarboPac PA 100 column (4 × 250 mm).

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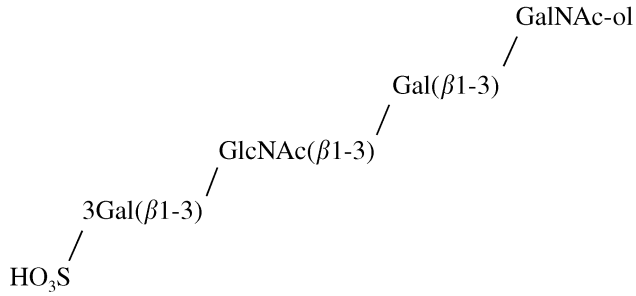


*Structures **IIIc-7A** and **IIIc-7B***

MALDI-MS analysis of fraction 7 shows the presence of a single ion $(M + 2Na\text{-}H)^+$ at m/z 1021 (Table 1), which may correspond to a sulfated pentasaccharide-alditol with GalNAc-ol, Gal, GlcNAc, Fuc and sulfate group in the molar proportion of 1:2:1:1:1. However the 1D ^{1}H-NMR spectrum of fraction 7 showed the presence of a mixture with structures of two core types: core 1 and core 2,

Table 1. MALDI-MS analysis of HPAEC-PAD-fractionated acidic oligosaccharide-alditols from fraction IIIc from non-secretor patient W.

Compound	*Molar ratio*						
	m/z[a]	*GalNAc-ol*	*Gal*	*GlcNAc*	*Fuc*	$-SO_3H$	Na^+
IIIc-3	no sugar						
IIIc-6	1021	1	2	1	1	1	2
IIIc-7	1021	1	2	1	1	1	2
IIIc-8	1224	1	2	2	1	1	2
IIIc-12	1021	1	2	1	1	1	2
IIIc-17	1224	1	2	2	1	1	2
IIIc-20	1224	1	2	2	1	1	2
IIIc-23′	875	1	2	1	–	1	2
IIIc-25	875	1	2	1	–	1	2
IIIc-29	1078	1	2	2	–	1	2

[a] $(M + 2Na\text{-}H)^+$ pseudomolecular ions of native oligosaccharide-alditols.

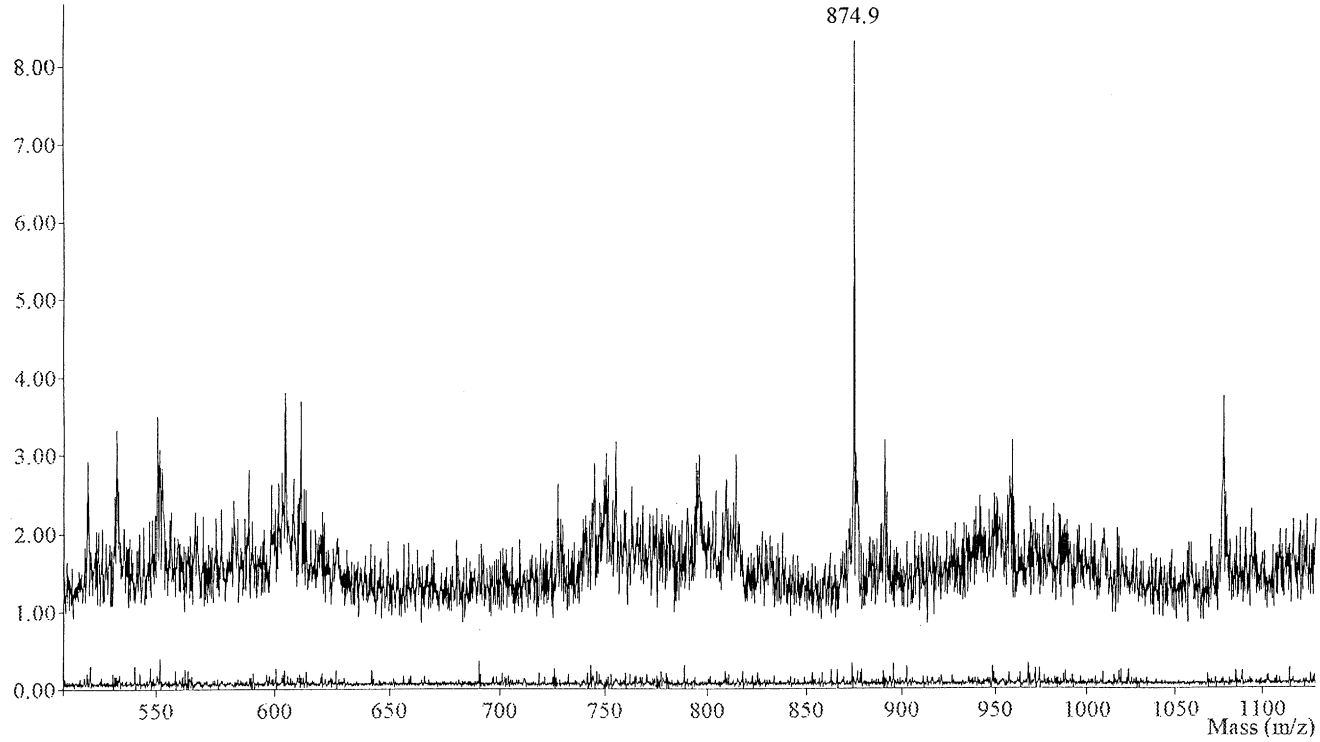
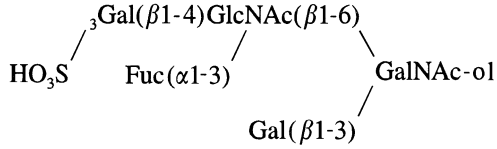


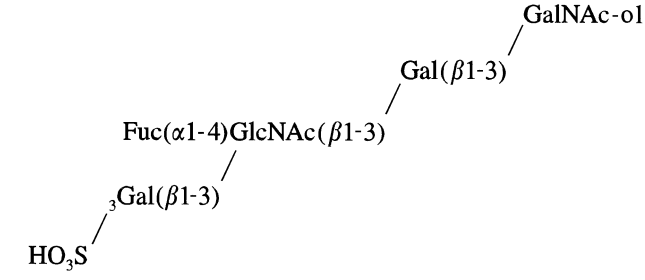
Figure 3. MALDI-MS analysis of fraction IIIc-25.

as shown by the characteristics of NMR parameters for Gal-NAc-ol (Table 2; Fig. 5). These structures were designated as IIIc-7a (core 2) and IIIc-7b (core 1). The proportion of structure IIIc-7a was higher than that of IIIc-7b. In the 1D ^{1}H-NMR spectrum, seven anomeric protons were visualized and, in order to elucidate these structures, it was necessary to realize a 2D-COSY spectrum, where the presence of eight anomeric protons was observed. The characteristics of this 2D-COSY spectrum allowed to determine the structure of 7a and 7b (Table 2; Fig. 5). Two fucose units linked to GlcNAc were clearly identified according to the set of their structural reporter group atom resonances (Fig. 5; and Table 2): One, F3, with an α1-3 linkage (δH1 = 5.113 ppm; δH5 = 4.810 ppm) and another, F4, with an α1-4 linkage (δH1 = 5.026 ppm; δH5 = 4.862 ppm). The H-2, H-3 and H-4 atom resonances of the GlcNAc units II′ (for isomer A) and III (for isomer B) are characteristic of such a monosaccharide unit respectively involved in the Le^x and Le^a determinants [17]. The chemical shifts of the H-3 and H-4 atoms of the Gal units III′ and IV are representative of O-3-sulfated residues [9]. The H-1 resonance of Gal III′, at δ = 4.565 ppm, is identical to that of Gal^{4,6} present in component IIIc2-9 [7], in which the sulfo-Le^x determinant is also present. The anomeric proton observed at δ = 4.482 ppm is correlated with two H-4 signals, one at δ = 3.899 ppm (Gal II$_A$, in terminal position), and the other at δ = 4.125 ppm (Gal II$_B$, O-3 substituted with GlcNAc III). Finally, the H-4, H-5 and H-6,6′ resonances of both GalNAc-ol IA and IB are in good accordance with the status of their O-3 and O-6 substitution (see Table 2; Fig. 5). In fact oligosaccharide 7a is an extension of the neutral saccharide 16a described in Klein *et al.* [18] and the structure 7b is the sulfated derivative of the oligosaccharide 14b described in Lhermitte *et al.* [3]. On the basis of these observations, the oligosaccharide-alditol 7a (core 2) and 7b (core 1) were defined as:

7a



7b



*Structure **IIIc-23′***

This was identified on the basis of its NMR and MALDI-MS parameters summarized in Tables 1 and 2. This compound

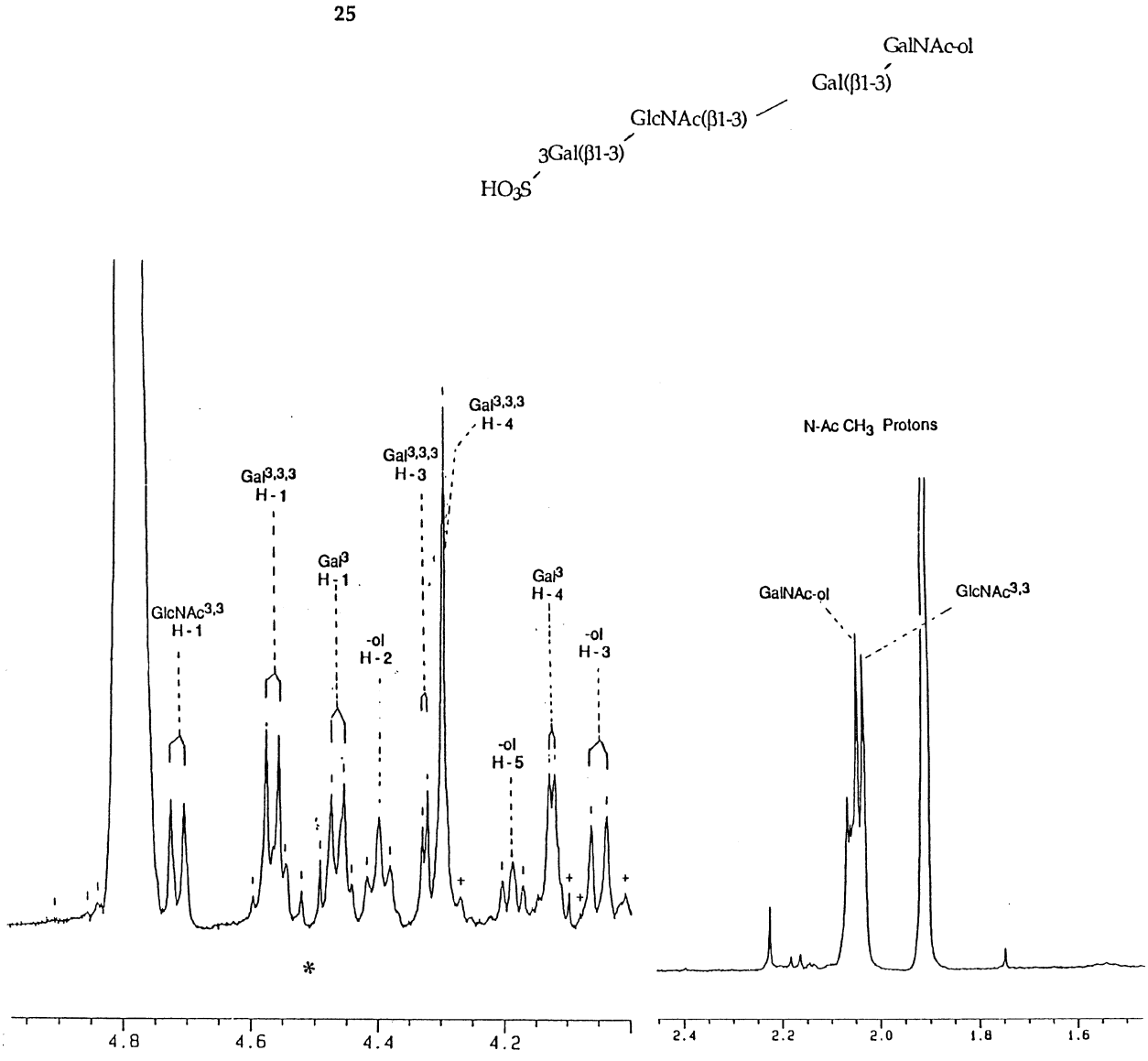
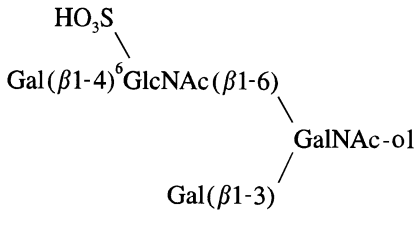


Figure 4. 400 MHz ^{1}H-NMR spectrum of fraction IIIc-25. The resonances marked by an * stem from frequently occurring non-protein, non-carbohydrate contaminants.

has been described previously in Hen ovomucin by Strecker *et al.* [19] and corresponds to the oligosaccharide IIIc2-20 observed in respiratory mucins from a patient with cystic fibrosis by Lo-Guidice *et al.* [7]. Its structure is:

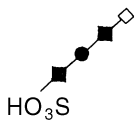
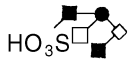
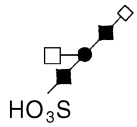
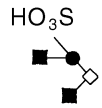
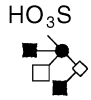
23'



*Structure **IIIc-12***

MALDI-MS analysis shows the presence of an ion $(M+2Na\text{-}H)^+$ at m/z 1021 (Table 1), which may correspond to a sulfated pentasaccharide-alditol with GalNAc-ol, Gal, GlcNAc, Fuc and sulfate group in the molar proportion of 1:2:1:1:1. The ^{1}H-NMR spectrum shows that this fraction contains an oligosaccharide-alditol with the core 2 type. This compound is an elongated structure of a structure already described: in fact, it is the (α1-3) fucosylated derivative of a pentasaccharide (IIIc2-20) described in Lo-Guidice *et al.* [7], and in this study (**IIIc-23'**). This is confirmed by the H-1 and CH_3 signals of the fucose residue ($\delta = 5.114$ and $\delta = 1.177$, respectively) (Table 2). It also

Table 2. ^{1}H chemical shifts of structural reporter groups of monosaccharides for the acidic oligosaccharide-alditols possessing the core unit, Gal(*β*1-3)GalNAc-ol.

Residue[a]	*Reporter group*	*Chemical shift in compound*[b]				
		IIIc-25 	*IIIc-7A* 	*IIIc-7B* 	*IIIc-23′* 	*IIIc-12* 
GalNAc-ol	H-1		3.73–3.76	3.73–3.76		
	H-2	4.400	4.394	4.394	4.394	4.38
	H-3	4.051	4.064	4.052	4.063	4.061
	H-4	nd	3.453	3.495	3.472	nd
	H-5	4.188	4.273	4.170	4.277	4.264
	H-6		3.626–3.928	3.62–3.63	–	–
	N-Ac	2.048	2.068	2.048	2.066	2.066
Gal^{3}	H-1	4.465	4.462	4.462	4.463	4.462
	H-2		3.569	3.590		
	H-3		3.669	3.725		
	H-4	4.126	3.899	4.125	3.898	nd
GlcNAc^{6}	H-1	–	4.560	–	4.584	4.594
	H-2	–	3.935	–		
	H-3	–	3.849			
	H-4	–	3.946			
	H-5	–	3.602			
	H-6/H6′	–	3.866–4.014	–	4.42/4.33	4.38
	N-Ac	–	2.048	–	2.066	2.057
Gal^{4,6}	H-1	–	4.565	–	4.543	4.548
	H-2	–	3.628	–		
	H-3	–	4.324	–	nd	nd
	H-4	–	4.267	–	3.928	nd
GlcNAc^{3,3}	H-1	4.716	–	4.686	–	–
	H-2	nd	–	3.965	–	–
	H-3	nd	–	4.118	–	–
	H-4	nd	–	3.764	–	–
	H-6	nd	–	nd	–	–
	N-Ac	2.036	–	2.056	–	–
Gal^{3,3,3}	H-1	4.556	–	4.622	–	–
	H-2	nd	–	3.620	–	–
	H-3	4.311	–	4.293	–	–
	H-4	4.297	–	4.277	–	–
Fuc^{3}	H-1	–	5.113		–	5.114
	H-2	–	3.682		–	
	H-3	–	3.903		–	
	H-4	–	3.786		–	
	H-5	–	4.810		–	nd
	CH_3	–	1.175		–	1.177
Fuc^{4}	H-1	–	–	5.026	–	–
	H-2	–	–	3.800	–	–
	H-3	–	–	3.891	–	–
	H-4	–	–	3.791	–	–
	H-5	–	–	4.862	–	–
	CH_3	–	–	1.182	–	–

[a] A superscript at a monosaccharide residue indicates to which position of the adjacent monosaccharide it is glycosidically linked. Two or three superscripts map out the pathway from the residue towards the GalNAc-ol residue.

[b] Chemical shifts are in p.p.m; relative to internal DSS in D_2O at 27 °C. The complete structures are listed in scheme 1. The table heading gives an abreviated version, in which the monosaccharides are denoted by the following symbols: ◇ = GalNAc-ol; ■ = Gal; ● = GlcNAc; □ = Fuc; the linkage positions are indicated by the angle of the connecting bars as follows: 

nd = not determined, – = absent in the considered structure.

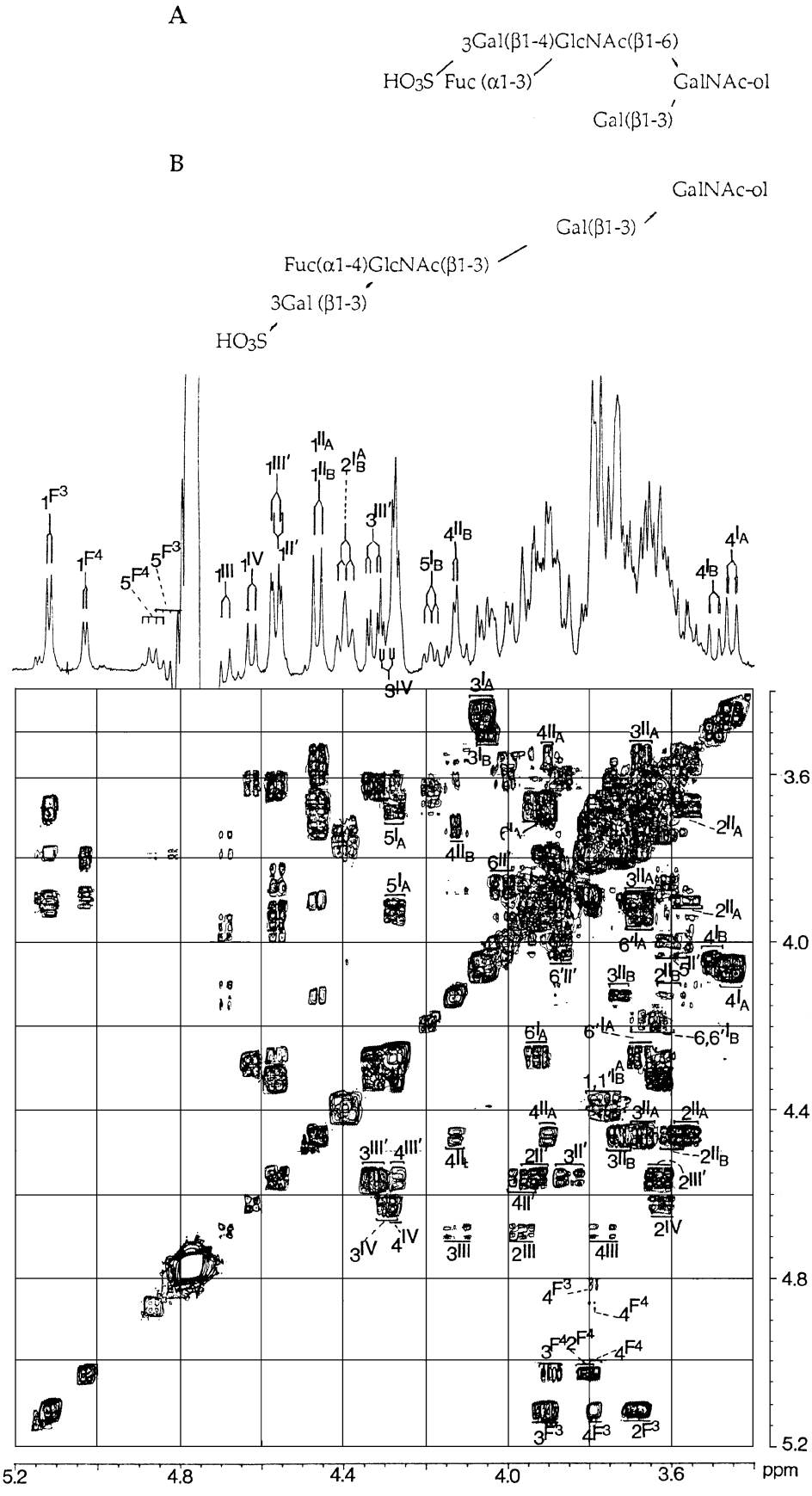
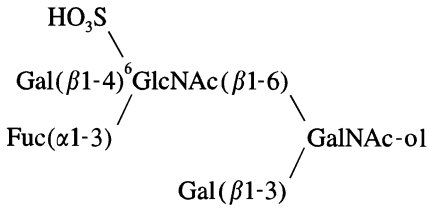


Figure 5. Two-dimensional homonuclear COSY spectrum of fraction IIIc-7.

corresponds to the sulfated derivative of a pentasaccharide described in Lhermitte *et al.* [3] in the same patient (15 + 16a), and previously observed by Klein *et al.* [18]. It is characterized by shifts of the H-6 and H-6′ of its *N*-acetylglucosamine residue typical for 6-sulfation [7, 20, 21]. In the ^{1}H-NMR spectrum, it is surprising to notice that the H-6 and H-6′ resonances of the GlcNAc^6 have a similar chemical shift (in fact a broad line) around $\delta = 4.38$ (Table 2), certainly due to the fact that the *N*-acetylglucosamine residue is completely substituted [7]. Therefore, the primary structure of IIIc-12 is the following:

12



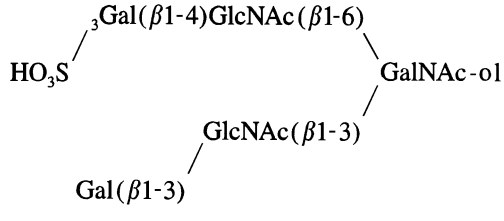
Oligosaccharide-alditols of core type 4

Structures with a core type 4 are recognized from the ^{1}H-NMR spectra by the GalNAc-ol H-2 signal at $\delta \approx 4.28$ and the H-5 signal at $\delta \approx 4.28$ [15]. Four structures were discovered to have this core (Table 3).

*Structure **IIIc-29***

MALDI-MS analysis shows the presence of an ion $(M + 2Na\text{-}H)^+$ at m/z 1078 (Table 1), which may correspond to a sulfated pentasaccharide-alditol with GalNAc-ol, Gal, GlcNAc and sulfate group in the molar proportion of 1:2:2:1. The NMR characteristics of this compound match those of oligosaccharide-alditol [15 + 16]b of this patient W., described in Lhermitte *et al.* [3] except for the Gal^{4,6} which has its H-1, H-3, H-4 resonating at $\delta = 4.587$, $\delta = 4.338$ and $\delta = 4.292$. These chemical shift values demonstrated the sulfation in C-3 of the galactose residue. This compound corresponds to the sulfated derivative of structure [15 + 16]b, found for the same patient by Lhermitte *et al.* [3]. Thus the structure proposed for IIIc-29 (structure 29) is:

29



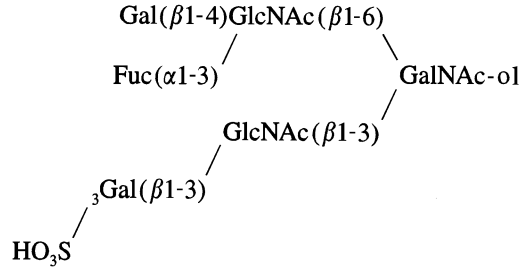
*Structures **IIIc-8** and **IIIc-17***

In both fractions IIIc-8 and IIIc-17 MALDI-MS analysis shows the presence of an ion $(M + 2Na\text{-}H)^+$ at *m/z* 1224 (Table I), which may correspond to a sulfated hexasaccharide-alditol with GalNAc-ol, Gal, GlcNAc, Fuc and sulfate group in the molar proportion of 1:2:2:1:1. The NMR characteristics of the two fractions clearly show the presence of a sulfate residue, either on a terminal galactose linked 1-4 to GlcNAc, or on a terminal galactose linked 1-3 to GlcNAc (Table 3). For one compound, the NMR characteristics match those of oligosaccharide-alditol 19a of this patient W., described in Lhermitte *et al.* [3] except for the Gal^{4,6} which has its H-1, H-3, H-4 resonating at $\delta = 4.566$, $\delta = 4.324$ and $\delta = 4.269$; chemical shift values demonstrated the sulfation in C-3 of this monosaccharide.

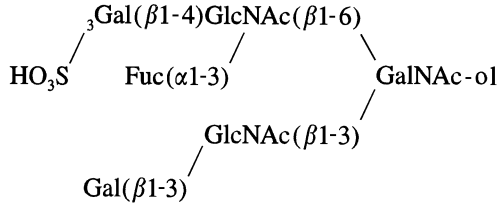
For the other compound, the NMR characteristics match those of oligosaccharide-alditol 19a of this patient W., described in Lhermitte *et al.* [3] except for the Gal^{3,3} which has its H-1, H-4 resonating at $\delta = 4.33$, and $\delta = 4.28$; chemical shift values demonstrated the sulfation in C-3 of this monosaccharide.

These findings determine the structures in fraction **IIIc-8** or **IIIc-17** as (Structure 8 or 17):

8 or 17



17 or 8

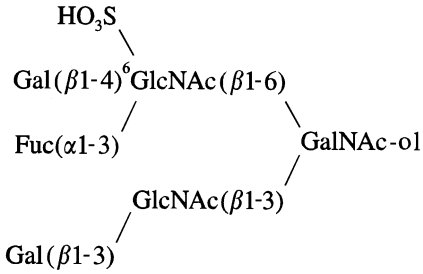


*Structure **IIIc-20***

MALDI-MS analysis shows the presence of an ion $(M + 2Na\text{-}H)^+$ at *m/z* 1224 (Table 1), which may correspond to a sulfated hexasaccharide-alditol with GalNAc-ol, Gal, GlcNAc, Fuc and sulfate group in the molar proportion of 1:2:2:1:1. This compound corresponds to the sulfated derivative of an hexasaccharide 19a found for the same non-secretor patient [3]. In the ^{1}H-NMR spectrum, it is surprising to notice that the H-6 and H-6′ resonances of the GlcNAc 6 have a similar chemical shift (in fact a broad line) around $\delta = 4.379$ (Table 3; Fig. 6), certainly due to the fact that the *N*-acetylglucosamine is completely substituted.

Therefore, the primary structure of **IIIc-20** is the following (Structure 20):

20

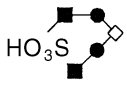
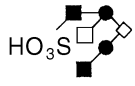
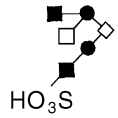
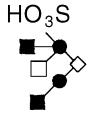


Discussion

Human respiratory mucins consist of families of very complex glycoproteins whose carbohydrate chains have been shown to be very heterogeneous with regard to their structure, molecular size and acidity [2]. So far, sulfated carbohydrate chains have been difficult to isolate in a very pure state with conventional techniques. In the present work, we have applied the protocol described by Lo-Guidice *et al.* [7], for the separation of acidic carbohydrate chains, based mainly on HPAEC. This procedure has allowed the structure determination of nine acidic sulfated carbohydrate chains from chronic bronchitis respiratory mucin glycopeptides, using high resolution ^{1}H-NMR spectroscopy in combination with MALDI-MS. Eight carbohydrate chains appeared to be novel structures. These carbohydrate chains are all monosulfated. Sulfation may occur either on the C-3 of a terminal galactose residue (in six carbohydrate chains) or on the C-6 of an N-acetylglucosamine residue (in three oligosaccharides).

^{1}H-NMR is known to be a suitable method to determine the presence and the exact location of sulfate in carbohydrate

Table 3. ^{1}H chemical shifts of structural reporter groups of monosaccharides for the HPAEC-PAD acidic oligosaccharide-alditols from a non-secretor patient and possessing the GlcNAc (β1-6)-[GlcNAc (β-3)]GalNAc-ol core unit.

Residue[a]	*Reporter group*	*Chemical shift in compound*[b]			
		IIIc-29 	*IIIc-17* 	*IIIc-8* 	*IIIc-20* 
GalNAc-ol	H-2	4.29	4.281	4.28	4.275
	H-3	4.04	nd	4.04	nd
	H-4	nd	nd	nd	nd
	H-5	4.29	4.221	4.20	4.177
	N-Ac	2.043	2.043	2.045	2.040
GlcNAc^3	H-1	4.655	4.648	4.637	4.654
	H-6	nd	4.008	nd	nd
	NAc	2.069	2.068	2.070	2.067
GlcNAc^6	H-1	4.546	4.569	4.568	4.601
	H-6/H6′	nd	4.003	nd	4.38/4.38
	N-Ac	2.062	2.053	2.053	2.053
Gal^{3,3}	H-1	4.453	4.452	4.33	4.552
	H-4	nd	nd	4.28	nd
Gal^{4,6}	H-1	4.587	4.566	4.46	4.553
	H-3	4.338	4.324	nd	nd
	H-4	4.292	4.269	nd	nd
Fuc^{3,6}	H-1	–	5.120	5.124	5.119
	H-5	–	nd	4.86	4.83
	CH_3	–	1.176	1.178	1.179

[a] A superscript at a monosaccharide residue indicates to which position of the adjacent monosaccharide it is glycosidically linked. Two or three superscripts map out the pathway from the residue towards the GalNAc-ol residue.

[b] Chemical shifts are in p.p.m; relative to internal DSS in D_2O at 27 °C. The complete structures are listed in scheme 1. The table heading gives an abbreviated version, in which the monosaccharides are denoted by the following symbols: ◇ = GalNAc-ol; ■ = Gal; ● = GlcNAc; □ = Fuc; the linkage positions are indicated by the angle of the connecting bars as follows: 

nd = not determined, – = absent in the considered structure.

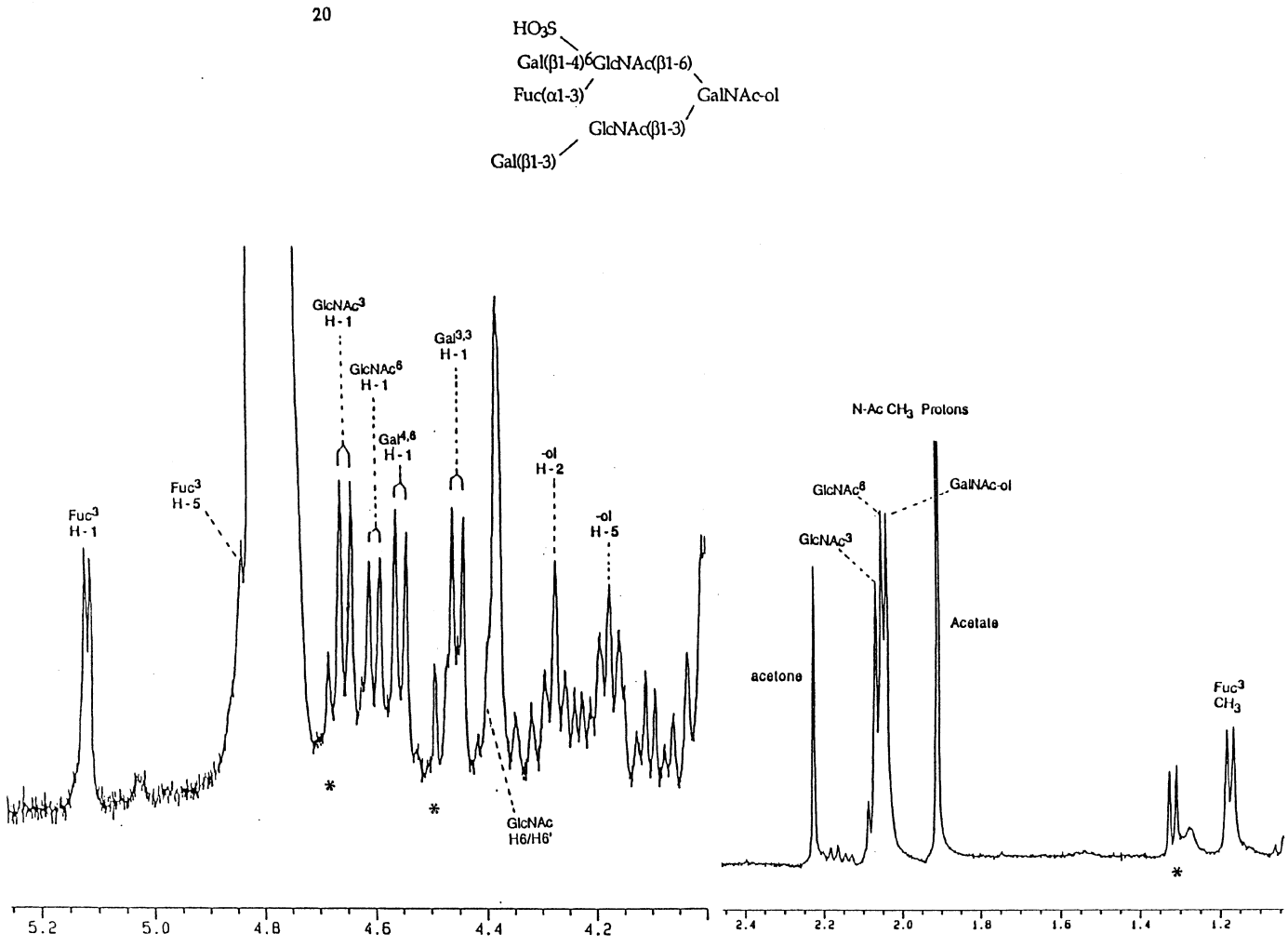


Figure 6. 400 MHz ^{1}H-NMR spectrum of fraction IIIc-20. The resonances marked by an * stem from frequently occurring non-protein, non-carbohydrate contaminants.

chains [20–23]. Sulfation of galactose at C-3 results mainly in a strong downfield shift for the H-1 and H-3 and H-4 protons. Sulfation of *N*-acetylglucosamine at C-6 results mainly in shifts of the H-6 and H-6′ of its *N*-acetylglucosamine residue. The present study confirms that, in mucus glycoproteins, sulfate may be attached to the C-3 of a terminal galactose [7, 9] or to the C-6 of a *N*-acetyl glucosamine residue [7, 10–12], implying at least two different sulfotransferases. A 3-sulfotransferase has been recently described by Lo-Guidice *et al.* [24]. We have not found any oligosaccharide with a terminal 6-sulfated *N*-acetylglucosamine, as also observed by Lo-Guidice *et al.* [7].

Structures of sulfated oligosaccharide-alditols in the mucins of CF patient have already been described, using conventional methods for structure determination, by Mawhinney and colleagues [10–12]. Sulfate was found either on C-6 of N-acetylglucosamine or on C-6, or C-4, of galactose, it was never observed on the C-3 of galactose. In this study, as in those of Lamblin *et al.* [9] and Lo-Guidice *et al.* [7] the only location of sulfate on galactose was on position 3. When comparing the studies of Mawhinney and colleagues [10–12], where ^{1}H-NMR has not been used for the structural determination, with the present study, we have not found any carbohydrate chains in common.

Most of these structures observed in the present study are an extension of structure found in the neutral fraction of oligosaccharide-alditols from the same patient [3].

In keeping with the non-secretor status of patient W, no structures among the IIIc subfractions were identified with a Fucα(1 → 2)Gal group. This observation strongly suggests that the α(1 → 2)-L-fucosyltransferase encoded by the Se gene is lacking in the respiratory mucosa of non-secretor individuals [25, 26]. The most complex structures found in

the present study correspond to oligosaccharides with sulfated Lewis X or Le^a determinants.

Sulfated carbohydrate chains are known to confer special chemical and physical properties to glycoconjugates, such as ion exchange and viscosity. Many studies have shown that such structures could be involved in biological phenomena, playing a role in recognition of microorganisms or cell adhesion molecules.

In vitro, *Mycoplasma pneumoniae*, a pathogen of the human respiratory tract binds to many receptors [27–29] and especially to HSO_3-3Galβ1-R sequences [28]. Such sequences, in human respiratory mucins might function in the trapping of these microbial pathogens allowing their elimination by the mucociliary clearance. Other microorganisms have been described to interact with sulfated structures of human cells [30, 31]. HSO_3-3Galβ1-R sequences are also ligands for selectins. 3-Sulfated galactosyl ceramides is a ligand of P-selectin [32]; E- and L-selectins have been proposed to bind Le^a/Le^x type tetrasaccharides sulfated at position 3 of outer galactose [33–35]. In the case of respiratory mucus, sulfated carbohydrate chains might favour the attachment of stimulated leukocytes to respiratory mucins.

The present study shows that, in the mucin of this chronic bronchitis patient, the sulfated oligosaccharides that have been isolated have core type 1, 2 or 4 and there are branched chains with one exception (structure 25). In the study of Lo-Guidice *et al.*. [7] most of the acidic oligosaccharides isolated had core 2 and were also branched oligosaccharides. When comparing the present study on oligosaccharides from a non-secretor patient suffering from chronic bronchitis with the study of Lo-Guidice *et al.* [7] on the acidic oligosaccharide-alditol of CF patient, only one oligosaccharide was found in common (oligosaccharide 23′). Moreover, in contrast to the series of oligosaccharides described by Lo-Guidice *et al.* [7], non-sialylated oligosaccharide was observed in the present series. We do not know if these differences have a clinical relevance. In the future, the comparison of the different carbohydrate chains in different pathologic states could be useful for determining the exact number of sulfotransferases acting in the human respiratory mucosa and for discovering the pathological alterations that may occur in various tracheobronchial diseases.

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